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A P P L I C A T I O N

for

UNITED STATES LETTERS PATENT

on

METHODS FOR TREATING DIABETES MELLITUS

by

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Number of Drawings: Two

Docket No.: SALK 1651
Salk File No.: S97037

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METHODS FOR TREATING DIABETES MELLITUS

RELATED APPLICATIONS

This application is a continuation-in-part of United States Serial No. 08/194,468, filed April 11, 1994, now pending, incorporated by reference herein in its entirety.

ACKNOWLEDGMENT

This invention was made in part with Government support under Grant No. GM 37828 provided by the National Institutes of Health. The Government may have certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates to analytical methods. In a particular aspect, the present invention relates to methods for the identification of compounds which mediate the interaction between signal dependent transcription factors and co-factor protein(s) involved in the activation of transcription. In another aspect, the present invention relates to methods for the identification of new signal dependent transcription factors. In yet another aspect, the present invention relates to methods for the identification of novel co-factor protein(s) which mediate the interaction between signal dependent transcription factors and inducer molecules involved in the activation of transcription. In yet another aspect, the present invention relates to methods for treating diabetes mellitus.

BACKGROUND OF THE INVENTION

Many eukaryotic genes are regulated in an inducible, cell type-specific fashion. Genes expressed in response to heat shock, steroid/thyroid hormones, phorbol esters, cyclic adenosine monophosphate (cAMP), growth factors and heavy metal ions are examples of this class. The activity of cells is controlled by external signals that stimulate or inhibit intracellular events. The process by which an external signal is transmitted into and within a cell to elicit an intracellular response is referred to as signal transduction. Signal transduction is generally initiated by the interaction of extracellular factors (or inducer molecules, i.e., growth factors, hormones, adhesion molecules, neurotransmitters, and other mitogens) with receptors at the cell surface. Extracellular signals are transduced to the inner face of the cell membrane, where the cytoplasmic domains of receptor molecules contact intracellular targets. The initial receptor-target interactions stimulate a cascade of additional molecular interactions involving multiple intracellular pathways that disseminate the signal throughout the cell.

Many of the proteins involved in signal transduction contain multiple domains. Some of these domains have enzymatic activity and some of these domains are capable of binding to other cellular proteins, DNA regulatory elements, calcium, nucleotides, lipid mediators, and the like.

Protein-protein interactions are involved in all stages of the intracellular signal transduction process - at the cell membrane, where the signal is initiated in the cytoplasm by receptor recruitment of other cellular proteins, in the cytoplasm where the signals are disseminated to different cellular locations, and in the

nucleus where proteins involved in transcriptional control congregate to turn on or turn off gene expression.

Mitogenic signaling affects the transcriptional activation of specific sets of genes and the inactivation of others. The nuclear effectors of gene activation are transcription factors that bind to DNA as homomeric or heteromeric dimers. Phosphorylation also modulates the function of transcription factors, as well. Oncogenes, first identified as the acute transforming genes transduced by retroviruses, are a group of dominantly acting genes. Such genes, which are involved in cell division, encode growth factors and their receptors, as well as second messengers and mitogenic nuclear proteins activated by growth factors.

The binding of growth factors to their respective receptors activates a cascade of intracellular pathways that regulate phospholipid metabolism, arachidonate metabolism, protein phosphorylation, calcium mobilization and transport, and transcriptional regulation. Specific phosphorylation events mediated by protein kinases and phosphatases modulate the activity of a variety of transcription factors within the cell. These signaling events can induce changes in cell shape, mobility, and adhesiveness, or stimulate DNA synthesis. Aberrations in these signal-induced events are associated with a variety of hyperproliferative diseases ranging from cancer to psoriasis.

The ability to repress intracellular signal-induced response pathways is an important mechanism in negative control of gene expression. Selective disruption of such pathways would allow the development of therapeutic agents capable of treating a variety of disease states related to improper activation and/or expression of specific transcription factors. For example, in patients

with non-insulin dependent diabetes mellitus (NIDDM), hyperglycemia develops, in part as a result of β cell failure secondary to chronic insulin resistance. This hyperglycemia appears to be exacerbated by hyperglucogonemia and increased hepatic gluconeogenesis. cAMP appears to be the major starvation state signal which triggers glucagon gene expression as well as transcription of PEPCK, the rate limiting enzyme in gluconeogenesis.

There remains, thus, a need in the art for selective disruption of intracellular signal-induced response pathways.

SUMMARY OF THE INVENTION

In accordance with the present invention, it has been discovered that CREB binding protein (CBP) cooperates with upstream activators involved in the activation of transcription by signal dependent transcription factors, such as c-Jun (responsive to phorbol ester), serum response factor, and the like. Accordingly, assays employing CBP have been developed for the identification of compounds which disrupt the ability of signal dependent transcription factors to activate transcription. In another aspect, assays employing CBP have been developed for the identification of new signal dependent transcription factors. In yet another aspect of the present invention, assays employing CBP have been developed for the identification of novel co-factor protein(s) which mediate the interaction between signal dependent transcription factors and inducer molecules involved in the activation of transcription. In still another aspect, an assay is provided to identify compounds which have the binding and/or activation properties characteristic of CREB binding protein. In still another aspect, methods employing compounds which inhibit intracellular signal-induced

response pathways have been developed for the treatment of diabetes mellitus.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a bar graph summarizing the injections described in Example 2. Each bar represents the percentage of positive cells expressing β-galactosidase from 2-3 experiments where 100-200 cells were injected in each experiment. [anti-CBP] denotes concentration of affinity purified CBP antiserum injected into cells. Right (hatched bars) indicate the percent lacZ positive cells after microinjection of CRE-lacZ reporter with CBP antiserum (anti-CBP) or control IgG (RbIgG). Preincubation of antisera with CBP peptide or non-specific ILS peptide (1mg/ml) was carried out as indicated.

Figure 2 is a bar graph summarizing the results of CBP antisera injections, as described in Example 3. Bars represent the percentage of lacZ positive (blue) cells (mean ± standard deviation) from 3-5 experiments where 100-200 cells were injected in each experiment. Injected cells were identified by immunofluorescence and/or lacZ staining. Reporter plasmid encoding the lacZ reporter was microinjected into NIH3T3 cells. CRE-, SRE-, TRE-lacZ reporter activities were determined after microinjected cells were treated as described herein. CMV-, RSV-, and SV40-lacZ reporter activities were measured in the absence of inducers. Hatched bars indicate % blue cells after microinjection with CBP antiserum. Solid bars indicate % blue cells following injection with control rabbit IgG (RbIgG).

DETAILED DESCRIPTION OF THE INVENTION

Cyclic AMP (cAMP) regulates the transcription of numerous genes through protein kinase-A (PK-A) mediated

phosphorylation, at Ser133, of transcription factor CREB. Within the CREB protein, a 60 amino acid Kinase Inducible Domain (KID) mediates transcriptional induction by PK-A. Based on recent work describing a nuclear CREB Binding Protein (CBP), it has been examined whether CBP is necessary for cAMP regulated transcription. Within CBP, a CREB binding domain has been identified, referred to as KIX which specifically interacts with the phosphorylated KID domain of CREB. About 600A of solvent accessible surface area in each protein is directly involved in formation of CREB:CBP complex. Phosphorylated Ser133 coordinates with a single arginine residue (Arg-600). The apparent Kd of the CREB:CBP complex is 0.4 μM.

Antisera against CBP have been found to completely inhibit transcription from a cAMP responsive promoter, but not from constitutively active promoters. Surprisingly, CBP has also been found to cooperate with upstream activators involved in phorbol ester and serum responsive transcription. It is demonstrated herein that recruitment of CBP to certain inducible promoters is intimately involved in transmitting inductive signals from phosphorylated, and thus activated, upstream factors to the RNA polymerase II complex. A number of analytical uses for CBP and CBP-like compounds based on these observations are described herein.

In accordance with the present invention, there is provided a method for the identification of a compound which inhibits activation of cAMP and mitogen responsive genes, said method comprising:

monitoring expression of reporter in response to exposure to said compound, relative to expression of reporter in the absence of said compound,

wherein exposure to said compound is carried out in the presence of:

a signal dependent transcription factor,

a polypeptide comprising at least amino acid residues 461-661 of the protein set forth in SEQ ID NO:2, and

a reporter construct comprising a reporter gene under the control of said signal dependent transcription factor.

As employed herein, the phrase "cAMP and mitogen responsive genes" refers to early response genes which are activated in response to a diverse array of agents including mitogens, such as, growth factors, differentiation inducers and biomodulators. Examples of such agents include insulin-like growth factor (IGF-1), erythropoietin (EPO), nerve growth factor (NGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor ß (TGFß), interferon, tumor necrosis factor (TNF), interleukins, granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, prolactin, serotonin, angiotensin, bombesin, bradykinin, noradrenalin, putrescine, concanavalin A, various oncogenic agents including tumor viruses, UV irradiation, estrogen, progesterone, testosterone, glucagon, PEPCK and the like.

Signal dependent transcription factors contemplated for use in the practice of the present invention include phosphorylation dependent activators such as CREB, Jun, Fos, and other early response genes such as Myc, Myb, erbA, and Rel, serum responsive factor, Elk, as well as steroid hormone receptors (e.g., glucocorticoid receptor (GR)), and the like.

Polypeptides employed in the invention assay function as co-factors by binding to the signal dependent transcription factor as a necessary component of a transcriptionally active complex. Examples of such co-factors include CBP (i.e., substantially the entire amino acid sequence set forth in SEQ ID NO:2), a polypeptide comprising amino acid residues 1-661 as set forth in SEQ ID NO:2, as well as functional fragments thereof, e.g., residues 461-661, and homologues thereof, such as those identified by the method described herein for the identification of compounds which have the binding and/or activation properties characteristic of CREB binding protein. In accordance with one embodiment of the present invention, there are provided purified and isolated polypeptides, CBPs, that bind to a specific sequence within phosphorylated CREB.

As used herein, the term "purified" means that the molecule is substantially free of contaminants normally associated with a native or natural environment. CREB binding protein, or functional fragments thereof, useful in the practice of the present invention, can be obtained by a number of methods, e.g., precipitation, gel filtration, ion-exchange, reversed-phase, DNA affinity chromatography, and the like. Other well-known methods are described in Deutscher et al., *Guide to Protein Purification: Methods in Enzymology* Vol. 182, (Academic Press, 1990), which is incorporated herein by reference.

Alternatively, a purified CBP, or functional fragment thereof, useful in the practice of the present invention, can also be obtained by well-known recombinant methods as described, for example, in Ausubel et al., *Current Protocols in Molecular Biology* (Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. 1993), also incorporated herein by reference. An example of recombinant means to prepare CBP, or functional fragments

thereof, is to express nucleic acid encoding CBP, or functional fragment thereof, in a suitable host cell, such as a bacterial, yeast or mammalian cell, using methods well known in the art, and recovering the expressed protein, again using methods well known in the art.

CBPs, and biologically active fragments thereof, useful in the practice of the present invention can also be produced by chemical synthesis. Synthetic polypeptides can be produced using Applied Biosystems, Inc. Model 430A or 431A automatic polypeptide synthesizer and chemistry provided by the manufacturer. CBP, and biologically active fragments thereof, can also be isolated directly from cells which have been transformed with the expression vectors described below in more detail.

The present invention also encompasses nucleic acids encoding CBP and functional fragments thereof. See, for example, SEQ ID NO:1. This invention also encompasses nucleic acids which encode substantially the entire amino acid sequence set forth in SEQ ID NO:2 (for example, the nucleic acid sequence set forth in SEQ ID NO:1, as well as nucleic acid sequences which differ from that set forth in SEQ ID NO:1 due to the degeneracy of the genetic code), nucleic acids which encode amino acid residues 1-661, as set forth in SEQ ID NO:2, nucleic acids which encode amino acid residues 461-661, as set forth in SEQ ID NO:2, as well as nucleic acids which encode substantially the same amino acid sequences as any of those referred to above, but which differ only by the presence of conservative amino acid changes that do not alter the binding and/or activation properties of the CBP or CBP-like polypeptide encoded thereby.

The invention further provides the above-described nucleic acids operatively linked to a promoter, as well as other regulatory sequences. As used herein, the

term "operatively linked" means positioned in such a manner that the promoter will direct the transcription of RNA from the nucleic acid. Examples of such promoters are SP6, T4 and T7.

Vectors which contain both a promoter and a cloning site into which a piece of DNA can be inserted so as to be operatively linked to the promoter are well known in the art. Preferably, these vectors are capable of transcribing RNA *in vitro* or *in vivo*. Examples of such vectors are the pGEM series (Promega Biotech, Madison, WI). This invention also provides a vector comprising a nucleic acid molecule such as DNA, cDNA or RNA encoding a CBP polypeptide. Examples of additional vectors useful herein are viruses, such as bacteriophages, baculoviruses and retroviruses, cosmids, plasmids, and the like. Nucleic acids are inserted into vector genomes by methods well known in the art. For example, insert and vector DNA can both be exposed to a restriction enzyme to create complementary ends on both molecules that base pair with each other and which are then joined together with a ligase. Alternatively, synthetic nucleic acid linkers that correspond to a restriction site in the vector DNA can be ligated to the insert DNA which is then digested with a restriction enzyme that recognizes a particular nucleotide sequence. Additionally, an oligonucleotide containing a termination codon and an appropriate restriction site can be ligated for insertion into a vector containing, for example, some or all of the following: a selectable marker gene, such as neomycin gene for selection of stable or transient transfectants in mammalian cells; enhancer/promoter sequences from the immediate early gene of human CMV for high levels of transcription; transcription termination and RNA processing signals from SV40 for mRNA stability; SV40 polyoma origins of replication and ColE1 for proper episomal replication; versatile multiple cloning sites; and T7 and SP6 RNA

promoters for *in vitro* transcription of sense and antisense RNA. Other means are available and can readily be accessed by those of skill in the art.

Also provided are expression vectors comprising DNA encoding a mammalian CBP, or functional fragment thereof, adapted for expression in a bacterial cell, a yeast cell, a mammalian cell or other animal cell. Such vectors comprise the regulatory elements necessary for expression of the DNA in the bacterial, yeast, mammalian or animal cells. Regulatory elements are positioned relative to the DNA encoding the CBP polypeptide so as to permit expression thereof. Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the lac promoter and the Shine-Dalgarno sequence and the start codon AUG (Ausubel et al., *supra* 1993) for transcription initiation. Similarly a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors can readily be obtained commercially or assembled by methods well known in the art, for example, the methods described above for constructing vectors in general. Expression vectors are useful to produce cells that express CBP or functional fragments thereof.

As employed herein, the term "reporter construct" refers to a recombinant construct, for example, an expression vector comprising a reporter gene under the control of a signal dependent transcription factor. In yet another example, the term refers to an expression vector comprising a reporter gene under the control of GAL4 response element. A compound which induces activation or inactivation of a target gene induces the reporter gene to

express an exogenous identifiable "signal". Expression of the reporter gene indicates that the target gene has been modulated. Exemplary reporter genes encode luciferase, β-galactosidase, chloramphenicol transferase, and the like.

In practicing the assays of the present invention, reporter plasmid is introduced into suitable host cells, along with CBP or a CBP-like polypeptide (or a DNA construct encoding same) and signal dependent transcription factor. The transfected host cells are then cultured in the presence and absence (as a control) of test compound suspected of being capable of inhibiting activation of cAMP and mitogen responsive genes. Next the transfected and cultured host cells are monitored for induction (i.e., the presence) of the product of the reporter gene.

In accordance with the present invention, expression of the reporter gene can be monitored in a variety of ways. Immunological procedures useful for *in vitro* detection of a polypeptide in a sample include immunoassays that employ a detectable antibody. Such immunoassays include, for example, ELISA, Pandex microfluorimetric assay, agglutination assays, flow cytometry, serum diagnostic assays and immunohistochemical staining procedures which are well known in the art. An antibody can be made detectable by various means well known in the art. For example, a detectable marker can be directly or indirectly attached to the antibody. Useful markers include, for example, radionuclides, enzymes, fluorogens, chromogens and chemiluminescent labels.

In accordance with still another embodiment of the present invention, there are provided methods to identify compounds which inhibit activation of cAMP and mitogen responsive genes, preferably compounds which

disrupt complex comprising CREB and CBP, said method comprising:

(a) contacting a modified host cell with a test compound, wherein said modified host cell comprises:

a first fusion protein comprising a GAL4 DNA binding domain, operatively associated with the KID domain of CREB,

a second fusion protein comprising an activation domain, operatively associated with the KIX domain of CBP, and

a reporter construct comprising a GAL4 response element operatively linked to a reporter gene; and

(b) selecting those test compounds which cause reduced expression of the reporter gene product.

In a preferred embodiment of the present invention, the first fusion protein comprises a GAL4 DNA binding domain, operatively associated with CREB and/or the second fusion protein comprises an activation domain operatively associated with CBP.

As used herein, the term "disrupt" embraces compounds which cause substantially complete dissociation of the various components of the complex, as well as compounds which merely alter the conformation of one or more components of the complex so as to reduce the repression otherwise caused thereby.

Any cell line can be used as a suitable "host" for the functional bioassay contemplated for use in the practice of the present invention. Thus, cells contemplated for use in the practice of the present

invention include transformed cells, non-transformed cells, neoplastic cells, primary cultures of different cell types, and the like. Exemplary cells which can be employed in the practice of the present invention include Schneider cells, CV-1 cells, HuTu80 cells, F9 cells, NTERA2 cells, NB4 cells, HL-60 cells, 293 cells, Hela cells, yeast cells, NIH3T3 cells and the like. Preferred host cells for use in the functional bioassay system are COS cells and CV-1 cells. COS-1 (referred to as COS) cells are monkey kidney cells that express SV40 T antigen (Tag); while CV-1 cells do not express SV40 Tag. The presence of Tag in the COS-1 derivative lines allows the introduced expression plasmid to replicate and provides a relative increase in the amount of receptor produced during the assay period. CV-1 cells are presently preferred because they are particularly convenient for gene transfer studies and provide a sensitive and well-described host cell system.

The above-described cells (or fractions thereof) are maintained under physiological conditions when contacted with physiologically active compound. "Physiological conditions" are readily understood by those of skill in the art to comprise an isotonic, aqueous nutrient medium at a temperature of about 37°C.

Various constructs employed in the practice of the present invention are well known in the art. Thus, the GAL4 DNA binding domain, the activation domain and GAL4 response elements have all been well characterized and extensively discussed in the art. For example, the DNA binding domain of the yeast GAL4 protein comprises at least the first 74 amino acids thereof (see, for example, Keegan et al., *Science* 231:699-704 (1986)). Preferably, the first 90 or more amino acids of the GAL4 protein will be used, with the first 147 amino acid residues of yeast GAL4 being presently most preferred.

Activation domains contemplated for use in the practice of the present invention are well known in the art and can readily be identified by the artisan. Examples include the GAL4 activation domain, BP64, VP16, and the like.

Exemplary GAL4 response elements are those containing the palindromic 17-mer:

5'-CGGAGGACTGTCCTCCG-3' (SEQ ID NO:4),

such as, for example, 17MX, as described by Webster et al., in Cell 52:169-178 (1988), as well as derivatives thereof. Additional examples of suitable response elements include those described by Hollenberg and Evans in *Cell* 55:899-906 (1988); or Webster et al. in *Cell* 54:199-207 (1988).

As used herein, the phrase "operatively associated with" means that the respective DNA sequences (represented, for example, by the terms "GAL4 response element" and "reporter gene") are operational, i.e., work for their intended purposes; the word "functionally" means that after the two segments are linked, upon appropriate activation by a ligand-receptor complex, the reporter gene will be expressed as the result of the fact that the corresponding "response element" was "turned on" or otherwise activated.

As readily recognized by those of skill in the art, the above-described assay can be modified to facilitate identification of compounds which inhibit any of the specific interactions involved in the formation of the CREB:CBP complex.

Compounds which are capable of inhibiting activation of cAMP and mitogen responsive genes, and hence can be identified by the invention assay method, include

antibodies raised against the binding domain of the protein set forth in SEQ ID NO:2, antibodies raised against the binding domain of CBP-like compounds, and the like. Presently preferred antibodies are those raised against a polypeptide fragment comprising amino acid residues from about 461 up to 661 of the protein set forth in SEQ ID NO:2; with antibodies raised against a polypeptide fragment comprising amino acid residues from about 634 up to 648 of the protein set forth in SEQ ID NO:2 (this subfragment is also set forth specifically as SEQ ID NO:3), being especially preferred. Alternatively, antibodies which are raised against the amino acid residues surrounding residue 600 of CBP (see SEQ ID NO:2) or antibodies which inhibit the phosphorylation of residue 133 of CREB are also desired (see, for example, Parker et al., Mol Cell Biol (1996) 16(2):694-703).

Antibodies contemplated for use in the practice of the present invention have specific reactivity with the above-described CBP or CBP-like compounds. Active antibody fragments are encompassed within the definition of "antibody." As used herein "specific reactivity" refers to the ability of an antibody to recognize and bind to an epitope on CBP or CBP-like compounds. Antibodies employed in the practice of the present invention can be produced by any method known in the art. For example, polyclonal and monoclonal antibodies can be produced by methods well known in the art, as described, for example, in Harlow and Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory 1988), which is incorporated herein by reference. The above-described CBP or CBP-like compounds can be used as the immunogen in generating such antibodies. Altered antibodies, such as chimeric, humanized, CDR-grafted or bifunctional antibodies can also be produced by methods well known to those skilled in the art. Such antibodies can also be produced by hybridoma, chemical or recombinant methodology described, for example in Ausubel

et al., *supra*. The antibodies can be used for determining the presence of a CBP-derived polypeptide, for the purification of CBP-derived polypeptides, for *in vitro* diagnostic methods, and the like.

Alternative compounds which are capable of inhibiting activation of cAMP and mitogen responsive genes include polypeptide fragments comprising amino acid residues from about 461 up to 661 of the protein set forth in SEQ ID NO:2. Polypeptide fragments comprising amino acid residues set forth specifically as SEQ ID NO:3 or KIX polypeptide fragments having a mutation at residue 600 (Arg-600), set forth in SEQ ID NO:2, are preferred, while KIX polypeptide fragments substituting Gln for Arg-600 are presently most preferred. Other polypeptide fragments contemplated for use in the practice of the present invention include those comprising the KID domain, preferably those comprising residue 133 of CREB. In the most preferred CREB polypeptide fragment, serine residue 133 is mutated to an amino acid residue which can not be phosphorylated. Other compounds which inhibit CREB activity (i.e., phosphorylated-Ser133) by binding to CBP include adenovirus E1A oncoprotein (Nakajima et al. Genes Dev (1997) 11(6):738-747), and the like. Those of skill in the art will readily recognize other polypeptide fragments which will readily inhibit the formation of CREB:CBP complex employing such assays as those disclosed herein.

In accordance with another embodiment of the present invention, there is provided a method for the identification of a compound which inhibits activation of cAMP and mitogen responsive genes, said method comprising:

(1) contacting a test system with said compound under physiological conditions; and

(2) monitoring expression of reporter in response to said compound, relative to expression of reporter in the absence of said compound, wherein said reporter is encoded by a reporter construct comprising a reporter gene under the control of a signal dependent transcription factor, and

wherein said test system comprises:

said signal dependent transcription factor,

a polypeptide comprising at least amino acid residues 461-661 of the protein set forth in SEQ ID NO:2, and

said reporter construct.

In accordance with yet another embodiment of the present invention, there is provided a method for the identification of a compound which promotes activation of cAMP and mitogen responsive genes, said method comprising:

monitoring expression of reporter in response to exposure to said compound, relative to expression of reporter in the absence of said compound,

wherein exposure to said compound is carried out in the presence of:

a signal dependent transcription factor, or

a polypeptide comprising at least amino acid residues 461-661 of the protein set forth in SEQ ID NO:2, and

a reporter construct;

wherein said reporter construct comprises a reporter gene under the control of a signal dependent transcription factor.

In accordance with still another embodiment of the present invention, there is provided a method for the identification of a compound which has the binding and/or activation properties characteristic of CREB binding protein, said method comprising:

monitoring expression of reporter in response to exposure to said compound, relative to expression of reporter in the absence of said compound,

wherein exposure to said compound is carried out in the presence of:

a signal dependent transcription factor, and

a reporter construct,

wherein said reporter construct comprises a reporter gene under the control of a signal dependent transcription factor.

In accordance with a still further embodiment of the present invention, there is provided methods for the identification of a compound which has the transcription activation properties characteristic of a signal dependent transcription factor, said method comprising:

monitoring expression of reporter in response to exposure to said compound, relative to expression of reporter in the absence of said compound,

wherein exposure to said compound is carried out in the presence of:

a polypeptide comprising at least amino acid residues 461-661 of the protein set forth in SEQ ID NO:2, and

a reporter construct,

wherein said reporter construct comprises a reporter gene under the control of a signal dependent transcription factor.

In accordance with a still further embodiment of the present invention, there are provided methods for treating diabetes mellitus, said method comprising contacting a biological system with an amount of an effective amount of a compound which inhibits binding of CREB to CBP. Such methods ameliorate hyperglycemia associated with diabetes mellitus by modulating gluconeogenesis and/or hyperglucagonemia. Particularly, such methods employ compounds which disrupt the formation of CREB:CBP complexes, thus inhibiting transcription of PEPCK or glucogon gene.

As employed herein, the phrase "biological system" refers to an intact organism or a cell-based system containing the various components required for response to the ligands described herein, e.g., an isoform of RAR (i.e., RARα, RARβ or RARγ), a silent partner for the RAR isoform (e.g., RXR), and an RAR-responsive reporter (which typically comprises an RAR response element (RARE) in operative communication with a reporter gene; suitable reporters include luciferase, chloramphenicol transferase, β-galactosidase, and the like.

Contacting in a biological system contemplated by the present invention can be accomplished in a variety of ways, and the treating agents contemplated for use herein can be administered in a variety of forms (e.g., in combination with a pharmaceutically acceptable carrier therefor) and by a variety of modes of delivery. Exemplary pharmaceutically acceptable carriers include carriers suitable for oral, intravenous, subcutaneous, intramuscular, intracutaneous, and the like administration. Administration in the form of creams, lotions, tablets, dispersible powders, granules, syrups, elixirs, sterile aqueous or non-aqueous solutions, suspensions or emulsions, and the like, is contemplated.

For the preparation of oral liquids, suitable carriers include emulsions, solutions, suspensions, syrups, and the like, optionally containing additives such as wetting agents, emulsifying and suspending agents, sweetening, flavoring and perfuming agents, and the like.

For the preparation of fluids for parenteral administration, suitable carriers include sterile aqueous or non-aqueous solutions, suspensions, or emulsions. Examples of non-aqueous solvents or vehicles are propylene glycol, polyethylene glycol, vegetable oils, such as olive oil and corn oil, gelatin, and injectable organic esters such as ethyl oleate. Such dosage forms may also contain adjuvants such as preserving, wetting, emulsifying, and dispersing agents. They may be sterilized, for example, by filtration through a bacteria-retaining filter, by incorporating sterilizing agents into the compositions, by irradiating the compositions, or by heating the compositions. They can also be manufactured in the form of sterile water, or some other sterile injectable medium immediately before use.

As employed herein, the phrase "effective amount" refers to levels of compound sufficient to provide circulating concentrations high enough to modulate the expression of gene(s) mediated by members of the steroid/thyroid superfamily of receptors. Such a concentration typically falls in the range of about 10 nM up to 2 μM; with concentrations in the range of about 100 nM up to 500 nM being preferred. Since the activity of different compounds described herein may vary considerably, and since individual subjects may present a wide variation in severity of symptoms, it is up to the practitioner to determine a subject's response to treatment and vary the dosages accordingly.

The invention will now be described in greater detail by reference to the following non-limiting examples.

EXAMPLE I

Functional Properties of CBP

To characterize the functional properties of CBP, rabbit CBP antiserum was developed against a fragment of CBP extending from amino acid residues 634-648 within the CREB binding domain of CBP (i.e., KVEGDMYESANSRDE; SEQ ID NO:3). Crude antiserum was affinity purified on a synthetic CBP peptide column, as described by Gonzalez et al., in *Mol. and Cell Biol.* 11(3):1306-1312 (1991), which is incorporated herein by reference. Far-Western and Western blot assays were performed as described by, for example, Chrivia et al., in *Nature* 365:855-859 (1993), also incorporated herein by reference. Thus, Western (CBP) and Far-Western (^{32}P-CREB) blot analysis of partially purified CBP protein from HeLa nuclear extract was carried out following SDS-PAGE and transfer to nitrocellulose. Far-Western blots were also obtained for crude HeLa nuclear extracts using ^{32}P-labeled CREB, phosphorylated with PK-A or casein kinase II (CKII). Far-Western blot analysis was also conducted with immunoprecipitates prepared from HeLa nuclear extracts with control IgG or affinity purified CBP antiserum (CBP-Ab). CREB binding activity was detected with ^{32}P-labeled CREB phosphorylated with PK-A.

Using the above-described antiserum, a 265 kD polypeptide was detected on Western blots, as predicted from the cDNA (see Chrivia et al., *supra*), which coincided with the predominant phospho-CREB binding activity in HeLa nuclear extracts by "Far-Western" blot assay. An identical phospho-CREB binding activity was also found in NIH3T3 cells. This phospho-CREB binding protein appeared to be specific for Ser133 phosphorylated CREB because no such band was detected with CREB labeled to the same specific

activity at a non-regulatory phospho-acceptor site (Ser156) by casein kinase II (CKII) (see Hagiwara et al., *Cell* 70:105-113 (1992), which is incorporated herein by reference).

To further demonstrate that the major phospho-CREB binding protein in HeLa and NIH3T3 cells is specifically bound by the anti-CBP antibody, immunoprecipitates were prepared from crude nuclear extracts using the CBP antiserum. Far-Western analysis of these immunoprecipitates revealed a 265 kD band in samples incubated with CBP antiserum, but not with control IgG.

EXAMPLE II

Role of Phosphorylation in CREB-CBP Interaction

To examine whether the phosphorylation dependent interaction between CREB and CBP was critical for cAMP responsive transcription, a microinjection assay was employed using CBP antiserum, which would be predicted to impair formation of a CREB-CBP complex. Thus, NIH3T3 cells were cultured in 5% CO_2 atmosphere in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum. Forty-eight hours prior to injection, cells were passaged into scored glass coverslips and made quiescent by incubation in medium containing 0.05% fetal calf serum for 24 hours (see, for example, Hagikara et al., *supra* and Alberts et al., in *Mol. and Cell Biol.* 13:2104-2112 (1993), both incorporated herein by reference). Representative fields of NIH3T3 cells were injected with pCRE-lacZ reporter plasmid plus 5, 0.5, and 0.05 mg/ml of affinity purified CBP antiserum. Total antibody concentration in microinjected cells was maintained at 5 mg/ml by adjusting with control Rabbit IgG. Injected cells were stimulated with 0.5 mM 8-bromo-cAMP, plus 3-isobutyl-1-methylxanthine (IBMX) for 4 hours, then fixed and assayed for lacZ

activity (β-Gal) as well as antibody content (Texas Red anti-Rb).

Following microinjection into nuclei of NIH3T3 cells, a CRE-lacZ reporter was markedly induced by treatment with 8-bromo-cAMP plus IBMX. Co-injection of CBP antiserum with the CRE-lacZ plasmid inhibited cAMP dependent activity in a dosage-dependent manner, but control IgG had no effect on this response.

To determine whether CBP antiserum inhibited cAMP responsive transcription by binding specifically to CBP, peptide blocking experiments were performed. Thus, the effect of CBP antiserum on CRE-lacZ reporter activity following pre-treatment of CBP antiserum with synthetic CBP peptide (anti-CBP+CBP) or unrelated peptide (anti-CBP+ILS; the unrelated peptide, ILS, is described by Leonard et al., in Mol. Endocr. 7: 1275-1283 (1993), which is incorporated herein by reference) was determined. Rabbit IgG+CBP and rabbit IgG pre-treated with CBP peptide were used as controls. NIH3T3 cells were injected with CRE-lacZ reporter plus various CBP antisera, stimulated with 0.5 mM 8-bromo-cAMP, plus IBMX for 4 hours, and assayed for lacZ activity. Cells expressing the lacZ gene product form a blue precipitate upon X-gal staining, which quenches immunofluorescent detection of the injected antibody.

CBP antiserum, pre-incubated with synthetic CBP peptide, was unable to recognize the 265 kD CBP product on a Western blot, and could not inhibit CRE-lacZ reporter activity upon microinjection into NIH3T3 cells. But antiserum treated with an unrelated synthetic peptide (ILS) retained full activity in both Western and microinjection assay, suggesting that the ability of the antiserum to bind CBP was critical for its inhibitory effect on cAMP dependent transcription.

Results of these experiments are summarized in Figure 1.

EXAMPLE III

Multiple Signaling Pathways Utilize CBP

To determine whether CBP activity may be restricted to a subset of promoters, several constitutively active reporter constructs were tested:

Cytomegalovirus (CMV-lacZ),
Rous sarcoma virus (RSV-lacZ), and
SV40 (SV40-lacZ).

Thus, cells were microinjected with CBP antiserum plus Rous Sarcoma Virus (pRSV-lacZ) or Cytomegalovirus (pCMV-lacZ) reporter constructs. Alternatively, NIH3T3 cells microinjected with CBP antiserum (or non-specific rabbit IgG (RbIgG)), plus reporter constructs containing either cAMP responsive elements (pCRE-lacZ), serum responsive elements (pSRE-lacZ) or phorbol ester responsive elements (pTRE-lacZ). Light field photo-micrographs show cells stained for β-galactosidase activity following four hour treatment with either 0.5 mM 8-bromo-cAMP, plus IBMX (pCRE-lacZ), 20% fetal calf serum (pSRE-lacZ), or 200ng/ml TPA (pTRE-lacZ). Results of β-galactosidase assays are summarized in Figure 2. Dark field photos show microinjected IgGs as visualized by immunofluorescence using Texas Red donkey anti-rabbit IgG.

When examined in NIH3T3 cells by transient transfection assay, each of the constitutively active reporter constructs had comparable basal activity, relative to the cAMP-stimulated CRE reporter plasmid, thereby permitting the effects of CBP antiserum on these reporters to be compared directly. Although co-injected CBP antiserum could block cAMP stimulated activity from a CRE-lacZ reporter in contemporaneous assays, no inhibition was observed on basal expression from any of the constitutive

promoter constructs tested, even when 10-fold lower amounts of reporter plasmid were employed.

These results suggest that CBP can indeed discriminate between basal and signal dependent activities *in vivo*.

EXAMPLE IV

CBP-involvement in non-CREB mediated pathways

Previous reports showing that serum and phorbol esters stimulate their target genes through phosphorylation-dependent trans-activators (see, for example, Hill et al., in *Cell* 73:395-406 (1993) or Smeal et al., in *Nature* 354:494-496 (1991), both incorporated herein by reference), suggested that CBP might also function in these signaling pathways. Thus, Far-Western analyses were carried out with crude HeLa nuclear extracts using ^{32}P-labeled recombinant Jun protein phosphorylated *in vitro* with either Jun-kinase (JNK; see Hibi et al., in *Genes and Develop.* 7:2135-2148 (1993), incorporated herein by reference) or casein kinase II (CK II).

Whereas serum and TPA could stimulate reporter activity in NIH3T3 cells microinjected with serum responsive element (SRE)-lacZ and TPA-responsive element (TRE)-lacZ indicator plasmids, respectively, co-injected CBP antiserum completely blocked both responses. These results suggest that CBP not only interacts with CREB, but also with other signal-dependent transcription factors.

In this regard, phorbol esters and serum induce TRE-dependent transcription, in part, through the Jun-kinase (JNK) mediated phosphorylation of c-Jun at Ser63 and Ser73 (see, for example, Smeal et al., *supra* or Hibi et al., *supra*). Using ^{32}P-labeled recombinant c-Jun protein,

phosphorylated at Ser63 and Ser73 with JNK, Far-Western blot assays were performed on crude HeLa nuclear extracts. JNK-phosphorylated c-Jun protein could bind CBP with comparable affinity to CREB. But c-Jun labeled to similar specific activity at non-activating sites (Thr 231, Ser243, and Ser249; see Boyle et al., in *Cell* 64:573-584 (1991)) by CKII, could not interact with CBP, suggesting that interaction between CBP and c-Jun requires phosphorylation of the transcriptionally active Ser63 and Ser73 phospho-acceptor sites. In view of the inhibitory effect of CBP antiserum on TRE-β gal reporter expression following phorbol ester and serum induction, the phosphorylation dependent interaction between CBP and c-Jun would appear to be a critical component of these response pathways.

EXAMPLE V

Chromatographic purification of CBP

Based on the surprising discovery that CBP cooperates with phosphorylation dependent activators by recruiting general transcription factors to target promoters, it was next examined whether CBP would co-fractionate with any general factors in HeLa nuclear extracts. Thus, Far-Western analyses of protein fractions were obtained after phospho-cellulose chromatography. Phospho-CREB binding proteins were visualized using ^{32}P-labeled CREB phosphorylated *in vitro* with PK-A (^{32}P-CREB). Western analysis was carried out with the same blot as described above, using affinity purified CBP antibody (CBP Ab). Far-Western (^{32}P-CREB) and Western (CBP-Ab) analyses of fractions were also carried out following DEAE and DE52 chromatography. Phosphocellulose, DEAE, and DE52 chromatography was performed on HeLa nuclear extracts as described by Ferreri et al., in *Proc. Natl. Acad. Sci. USA* in press (1993), which is incorporated herein by reference.

Both CBP-immunoreactive and phospho-CREB binding activities were retained on phosphocellulose columns and were eluted at 0.3-0.5M KCl. Further purification of a comparable phospho-cellulose fraction on DEAE-sepharose and DE52 resins showed that CBP was highly enriched in fractions containing TFII (E, F, H) but not TFIID activities. Although the general factor which associates directly with CBP is not known, the co-fractionation of CBP with proteins involved in basal transcription initiation suggests a testable mechanism for CBP action. In particular, the results presented herein suggest that phosphorylation-dependent activators like CREB and Jun influence assembly of late-acting factors (TFII E, F, H) during transcriptional initiation/reinitiation by interacting with CBP in a signal dependent manner.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.